Peptides 53 (2014) 134–139

Contents lists available at ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides

Identification of the short neuropeptide F precursor in the desert locust: Evidence for an inhibitory role of sNPF in the control of feeding

Senne Dillen, Rik Verdonck, Sven Zels, Pieter Van Wielendaele, Jozef Vanden Broeck*

Molecular Developmental Physiology and Signal Transduction, Department of Biology, KU Leuven, Belgium

ARTICLE INFO

Article history:
Received 26 August 2013
Received in revised form
27 September 2013
Accepted 27 September 2013
Available online 12 October 2013

Keywords:
sNPF
Short neuropeptide F
Precursor
Feeding
Locust
Neuropeptide
Insect
Satiety
Schistocerca

ABSTRACT

Peptides of the short neuropeptide F (sNPF) family have been shown to modulate feeding behavior in a wide variety of insect species. While these peptides stimulate feeding and food-searching behavior in *Drosophila melanogaster* and *Apis mellifera*, an opposite effect has recently been demonstrated in the desert locust, *Schistocerca gregaria*. In this study, we elaborate on these observations with the identification of the nucleotide sequence encoding the *Schgr*-sNPF precursor and the study of its role in the regulation of locust feeding behavior. We confirm that both *Schgr*-sNPF-like peptides, previously identified in mass spectrometric studies, are genuine precursor-encoded peptides. RNA interference mediated silencing of the *Schgr*-sNPF precursor transcript generates novel evidence for an inhibitory role of *Schgr*-sNPF in the regulation of feeding in *S. gregaria*. Furthermore, we show that starvation reduces the *Schgr*-sNPF precursor transcript level in the optic lobes, the primary visual centers of the locust brain. Our data indicate that *Schgr*-sNPF exerts an inhibitory effect on food uptake in the desert locust, which contrasts with effects of sNPF reported for several other insect species.

1. Introduction

Peptides of the short neuropeptide F (sNPF) family, characterized by a carboxyterminal xPxLRLRFamide sequence, show a widespread occurrence throughout the arthropod phylum and have been found in all arthropod species of which ample sequence data are available [22]. Interestingly, no sNPF-like peptides have been discovered in non-arthropod species, making the sNPF signaling pathway a prime candidate for further research exploring the use of neuropeptidergic signaling in the control of insect pests [11].

The sNPF peptides are cleaved from a larger prepropeptide precursor, containing an aminoterminal signal peptide which allows for transport of the precursor peptide to the endoplasmatic reticulum for further processing and for secretion to the extracellular environment. These sNPF prepropeptides typically yield multiple sNPF isoforms. In *Drosophila melanogaster*, for example, the sNPF precursor (sNPFp) encodes four *Drome*-sNPFs [31], while the sNPF prepropeptide of *Apis mellifera* yields two *Apime*-sNPF isoforms [12]. While these sNPF peptide precursors typically yield multiple sNPF isoforms, this is not the case in all insect species. In the kissing bug, *Rhodnius prolixus*, for example, only a single sNPF is cleaved from the *Rhopr*-sNPF prepropeptide [24].

In the desert locust, *Schistocerca gregaria*, the presence of two sNPF-like peptides has previously been confirmed by mass spectrometry. These peptides display a large overlap in amino acid sequence and at the time of their discovery, it could not be excluded that the smaller *Schgr*-sNPF$^{4-11}$ (SPSLRLRFamide) was only a degradation product of the larger *Schgr*-sNPF (SNRSPRLRLRFamide) [8]. Recent *in vivo* experiments, however, pointed out that both *Schgr*-sNPF and *Schgr*-sNPF$^{4-11}$ display physiological activity. Furthermore, both *Schgr*-sNPF peptides are potent ligands of the *Schgr*-sNPF receptor (sNPFR), capable of inducing both a rise of intracellular calcium ion levels and a decrease of intracellular cyclic AMP [10].

Mass spectrometric analysis showed that the *Schgr*-sNPF peptides are abundant throughout the central nervous system and their presence has been confirmed in the brain, *corpora cardiaca*, *corpora allata*, the recurrent and suboesophageal nerves and a variety of ganglia [9].

Although peptides of the sNPF family were demonstrated to play a role in a wide variety of physiological processes including molting [18], sleep homeostasis [7] and reproduction [5], their main function seems to lie in the regulation of feeding and food-searching behavior.

A first indication of these effects on feeding was obtained when overexpression of sNPF in *D. melanogaster* promoted food uptake

* Corresponding author.
E-mail address: jozef.vandenbroeck@bio.kuleuven.be (J. Vanden Broeck).

0196-9781/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.peptides.2013.09.018

and yielded larger and heavier flies. Conversely, flies in which the *Drome*-sNPFp transcript was knocked down, displayed the opposite phenotype. It should be noted, however, that these stimulatory effects on feeding were only observed in larvae in the feeding stage of development and that the feeding responses of larvae in the wandering stage did not differ from those observed in control animals [15]. Similar to the results obtained for *Drome*-sNPF, overexpression of the *Drosophila* sNPFR, *Drome*-sNPFR1, led to an increase in body size while RNA interference (RNAi) mediated knockdown of the receptor transcript yielded smaller animals when compared to wild-type controls. Interestingly, this reduction in *Drome*-sNPF signaling activity also led to an increase in lifespan, an effect that is likely mediated by the *Drosophila* insulin-like peptides [14].

A positive correlation between sNPF signaling and feeding, as demonstrated in *Drosophila*, was also observed in several other insect species. When sNPF and sNPFR transcript levels were studied in the honey bee, *A. mellifera*, both were found to be higher in foraging animals compared to animals providing brood care [2]. Furthermore, sNPF reduced the latency to feeding in the silk moth, *Bombyx mori*, rendering the animals more prone to feed [21]. sNPF and sNPFR expression levels were also higher in starved fruit flies [25] and cockroaches [19], suggesting a correlation with an increase in food-searching behavior. In *D. melanogaster*, this starvation-dependent upregulation of sNPF signaling was mapped to certain olfactory receptor neurons in the antennal lobes of the brain. Upon starvation, sNPF expression in these neurons increased and flies demonstrated augmented food-searching behavior. When sNPF signaling in these neurons was inhibited however, this starvation-dependent increase in food-searching behavior was abolished [25].

Although there seems to be a positive correlation between sNPF signaling and feeding in the fruit fly and the honey bee, this does not seem to be the case in all insect species. Early studies on the *Aedes aegypti* sNPF-like peptide, Head Peptide I (HP-I), showed that the levels of circulating peptide increased fivefold after a replete blood meal. Furthermore, injection of this *Aedae*-HP-I potently inhibited host-seeking behavior in adult female mosquitos [4]. In the red imported fire ant, *Solenopsis invicta*, transcript levels of the sNPF receptor were also shown to be higher in starved animals compared to non-starved controls [6].

Further indications were acquired in the silk moth, *Bombyx mori*. While injection of *Bommo*-sNPF-2 reduced the latency to feeding in silk moth larvae [21], Nagata et al. also showed that the expression levels of *Bommo*-sNPF-1 and -2 decreased upon starvation, a state in which the animals are more likely to engage in food-searching behavior [20].

Arguably the strongest indications for an inhibitory role of sNPF in the regulation of feeding were recently obtained in the desert locust, *S. gregaria*. In these studies, starvation resulted in a decrease of the *Schgr*-sNPF receptor transcript levels, while feeding led to a transient peak in the receptor transcript level. Furthermore, injecting either of the two *Schgr*-sNPF isoforms resulted in an augmentation of food-uptake. When sNPF signaling activity was reduced by RNAi-induced knockdown of the *Schgr*-sNPFR transcript, the opposite (stimulatory) effect was observed [10].

In this paper, we describe the identification of the *Schgr*-sNPF peptide precursor and discuss its role in the regulation of feeding behavior. We show that both *Schgr*-sNPF and *Schgr*-sNPF$^{4\text{-}11}$ are genuine *Schgr*-sNPF precursor-derived isoforms. Furthermore, we present more evidence for an inhibitory role of sNPF in the regulation of feeding. Silencing the *Schgr*-sNPF precursor mRNA, significantly increases food uptake, while starvation leads to a decrease in sNPF precursor transcript levels in the optic lobes, the primary visual centers of the locust brain.

2. Materials and methods

2.1. Identification and cloning of the Schgr-sNPF precursor cDNA

From a recently generated whole-body transcriptome database of *S. gregaria* (Verdonck et al., in preparation), a cDNA sequence encoding the two *Schgr*-sNPF isoforms was retrieved. This nucleotide sequence was amplified from brain-derived cDNA, using the following nucleotide primers: *Schgr*-sNPF-Fw: 5′-GTCCGCTGTGTGCAAGGT-3′, *Schgr*-sNPF-Rv: 5′-GTTGGATCGGAGCGTATTTC-3′ (Sigma–Aldrich) and the following program: 95 °C for 120 s, followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 120 s. After these cycles, the program was completed with a final elongation step of 72 °C for 120 s and ended at 10 °C. The PCR product was run on a 1% agarose gel, from which it was purified using the GenElute™ Gel Extraction Kit (Sigma–Aldrich). Amplicons were then cloned in a pCR®2.1-TOPO® vector (Invitrogen), which was used to transform One Shot TOP10 chemically competent *Escherichia coli* cells (Invitrogen). These were plated on LB agar plates (35 g/l, Sigma–Aldrich) with ampicillin (10 mg/ml, Invitrogen) and grown overnight at 37 °C. Colonies were collected and transferred to LB medium (25 g/l, Sigma–Aldrich) with ampicillin (10 mg/ml, Invitrogen) and again grown overnight at 37 °C. Plasmids were extracted using the GenElute™ HP Plasmid Miniprep kit (Sigma–Aldrich) and the sequences of the insert fragments were verified on a ABI PRISM 3130 Genetic Analyzer (Applied Biosystems) using the ABI PRISM BigDye Terminator Ready Reaction Cycle Sequencing Kit (Applied Biosystems).

2.2. Animal rearing conditions

All animals used in this study were gregarious adult locusts. They were reared under crowded conditions (over 200 locusts per cage) at constant temperature (32 ± 1 °C) and photoperiod (L/D: 14 h/10 h). During rearing, they were fed daily with cabbage and dry oat flakes. The locusts used in the feeding experiment were placed in separate cages and placed on a controlled dietary regimen one week prior to the assay in order to normalize feeding responses. In this controlled regimen animals were allowed to feed *ad libitum* for 2 h each day (9–10 h, 16–17 h). The animals used in starvation studies were placed in separate cages and deprived of food for a period of five days. Non-starved control animals were also placed in these cages but were allowed to feed *ad libitum*.

2.3. Schgr-sNPF precursor transcript distribution

Locust tissues were dissected under a binocular microscope and collected in MagNa Lyser Green Beads (Roche). The dissected tissues were immediately frozen in liquid nitrogen and stored at −80 °C. Prior to RNA extraction, the tissues were homogenized using the MagNa Lyser instrument. RNA extraction was performed using the RNeasy® Lipid Tissue Mini Kit (Qiagen), in combination with a DNase digestion of the purified nucleic acids (RNase-free DNase Set, Qiagen), or the RNaqueous®-Micro Kit (Ambion). The resulting total RNA was reverse transcribed to cDNA using the PrimeScript™ RT Reagent Kit (Takara). The resulting cDNA was diluted twentyfold and used as template in quantitative (realtime) reverse transcription PCR (qRT-PCR).

The primers used in the qRT-PCR analysis were designed by means of the Primer Express® software (Applied Biosystems, Table 1) and subjected to melting curve analysis to verify their specificity and efficiency of amplification. Amplification products were also analyzed by means of electrophoresis on a 1% agarose gel. Visual inspection of the PCR products showed a single band of the expected size. Additionally, the PCR products were sequenced

Table 1
Primer sequences for the qRT-PCR assays.

	Forward primer (5′–3′)	Reverse primer (5′–3′)
Schgr-sNPF	TCCCAGCTACCCAGACTATGACA	GCCTCGCGCTGCAGAA
Schgr-EF1α	GATGCTCCAGGCCACAGAGA	TGCACAGTCGGCCTGTGAT
Schgr-RP49	CGCTACAAGAAGCTTAAGAGGTCAT	CCTACGGCGCACTCTGTTG

to further verify the specificity of the qRT-PCR amplification. The reference genes used in the study were analyzed using the geNorm applet [32], revealing ribosomal protein 49 (RP49) and Elongation Factor 1α (EF1α) to be the most stably expressed pair over the sample set. For qRT-PCR, we used Fast SYBR® Green Master Mix (Applied Biosystems), as per manufacturer's instruction, and the StepOnePlus™ Real-Time PCR system (Applied Biosystems). The Fast SYBR® Green Master Mix contains the fluorescent ROX™, which is used as a passive reference. All samples were measured in duplicate and all plates contained a no template control for all three primer pairs to check for possible contaminations in the master mix. The following program was used: 95 °C for 10 min, followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. Data were analyzed using the $\Delta\Delta C_T$ method, in which the cycle threshold (C_T) value for the gene of interest (GOI) is normalized to the C_T of one or more reference genes (ΔC_T) and to the $C_{T\,GOI}$ of a calibrator sample ($\Delta\Delta C_T$), included on each plate [16].

2.4. Schgr-sNPF precursor transcript knockdown

T7 promoter sites were incorporated on either side of a 256 bp fragment of the *Schgr*-sNPF precursor cDNA. This was done by performing PCR, using the following primers: *Schgr*-sNPF-T7-Fw 5′-TAATACGACTCACTATAGGGAGACTGGTGCGCAAGTCCAAC-3′ and *Schgr*-sNPF-T7-Rv 5′-TAATACGACTCACTATAGGGAGATTCTCCACTGGACTCTCTGGA-3′ (Sigma–Aldrich) and the following program: 95 °C for 120 s, followed by 10 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 60 s, and 15 cycles of 95 °C for 30 s, 65 °C for 30 s and 72 °C for 60 s. After these cycles, the program was completed with a final elongation step of 72 °C for 120 s and ended at 10 °C. Amplicons were cloned into a pCR®2.1-TOPO® vector (Invitrogen), used in the transformation of One Shot TOP10 chemically competent Escherichia coli cells (Invitrogen), and sequenced, as described above for the *Schgr*-sNPF precursor cDNA.

Double stranded RNA (dsRNA) was generated using the MEGAscript® RNAi Kit (Ambion) and its length was checked on a 1% agarose gel. The efficiency of the knockdown was assayed by injecting adult locusts with 2 μl of a 100 ng/μl dsRNA solution, the dsRNA corresponding to either *Schgr*-sNPF (experimental) or GFP (control). Animals were injected dorsally, between the first and second abdominal segment, using a 710 RN injector (Hamilton). Five and eight days after injection, locust brains (including the optic lobes) were dissected. Since *S. gregaria* shows a highly potent and systemic RNAi response, a knockdown of the *Schgr*-sNPFp transcript in this tissue will also be indicative of effects on other tissues. The high efficiency of RNAi in multiple tissues of the desert locust has previously been demonstrated in a number of studies and has been studied in detail by Wynant et al. [17,27,28,34]. Total RNA was extracted from the dissected tissues and was reverse transcribed into cDNA and used as template in qRT-PCR analysis, as described previously for the *Schgr*-sNPF precursor transcript distribution.

2.5. Feeding assay

Two groups of age- and sex-matched locusts were injected with 2 μl of a 100 ng/μl solution of dsRNA, corresponding to either *Schgr*-sNPFp or GFP. The feeding assay was performed five days after injection, at the time of the first daily feeding session. Pieces of cabbage leaf were weighed in advance and presented to the locusts for 1 h, after which all animals had stopped feeding. The pieces of cabbage were then weighed again and the total food uptake was determined. In parallel, similar pieces of cabbage leaf were put in empty cages during the course of the experiment, allowing for a correction for weight-loss due to evaporation. The results were analyzed and visualized using GraphPad Prism 5 (GraphPad Software).

3. Results

3.1. Identification of the Schgr-sNPF precursor

Using the peptide sequences of the two *Schgr*-sNPF isoforms as a query, we were able to identify the sNPF precursor sequence in a recently developed whole-body transcriptome database of *S. gregaria*. The precursor's nucleotide sequence was cloned and the confirmed sequence was uploaded to Genbank [GenBank: KF360057]. The region encoding the two sNPF isoforms is flanked by a start- and stop-codon, indicating that the complete coding sequence is represented (Fig. 1). At the aminoterminal end of the precursor peptide, the presence of a secretory signal peptide is predicted (InterProScan, EMBL European Bioinformatics Institute). The amino acid sequences of *Schgr*-sNPF and *Schgr*-sNPF$^{4\text{-}11}$ are both present in the precursor sequence and each of these is flanked by mono- or dibasic cleavage sites and followed by an amidation signal.

3.2. Schgr-sNPF precursor transcript distribution

Quantitative reverse transcription PCR was used to study *Schgr*-sNPFp transcript levels in a wide variety of tissues. The transcript was primarily detected in the central nervous system, with only a very limited expression in the flight muscles and the male and female gonads. The brain, optic lobes, and *corpora cardiaca* proved to be the major sites of *Schgr*-sNPFp mRNA expression. Lower transcript levels were detected in the *corpora allata* and the suboesophageal, thoracic, and abdominal ganglia (Fig. 2). The effect of starvation on sNPFp transcript levels was studied in the brain, the optic lobes, the *corpora cardiaca* and the suboesophageal ganglion. Starvation was shown to reduce *Schgr*-sNPFp transcript levels in the optic lobes by 35%, while no significant differences were detected in the other three tissues that were analyzed (Fig. 3).

3.3. Knockdown of Schgr-sNPF transcript levels stimulates food uptake

To further study the functional role of sNPFs in the desert locust, we knocked down the *Schgr-sNPFp* transcript by means of RNA interference. This procedure yielded a potent reduction of the abundance of *Schgr*-sNPFp transcripts. Five days after injection, *Schgr*-sNPFp transcript levels in the brain showed a decrease of over 90% when compared to control animals injected with dsRNA corresponding to the mRNA corresponding to GFP. This 90%

MASTSAVCKVALVLLLVAALAASAPSYPDYDNVRDLYELLLQREAEGARLAAAADDHQLVRK**SNRSPSLRLRF**GRRSDP
LFGAPTAAAGSGQDSLAVAAR**SPSLRLRF**GRRSDPLLSNQLGAPESPVEN-

Fig. 1. *Schgr*-sNPF precursor sequence. Both known isoforms of *Schgr*-sNPF are encoded in the precursor sequence, flanked by mono- or dibasic cleavage sites and followed by an amidation signal. Underlined: signal peptide, bold: peptide sequence, light gray: cleavage sites, and dark gray: amidation signal.

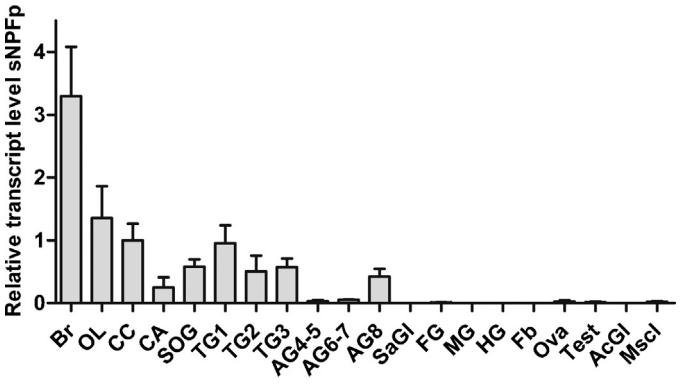

Fig. 2. *Schgr*-sNPF precursor transcript distribution in adult locusts. The occurrence of the *Schgr*-sNPFp mRNA is limited to the central nervous system. Only basal expression is detected in some non-neuronal tissues. Br: brain; OL; optic lobes; CC: corpora cardiaca; CA: corpora allata; SOG: suboesophageal ganglion; SaGl: salivary gland; TG1: prothoracic ganglion; TG2: metathoracic ganglion; TG3: metathoracic ganglion; Ova: ovaries; Test: testes; Fb: fatbody; FG: foregut; MG: midgut; HG: hindgut; AcGl: male accessory gland; and Mscl: flight muscle. Data represent the average ± SEM (data acquired from three independent pools of adult animals with 40, 10, 10 locusts/pool).

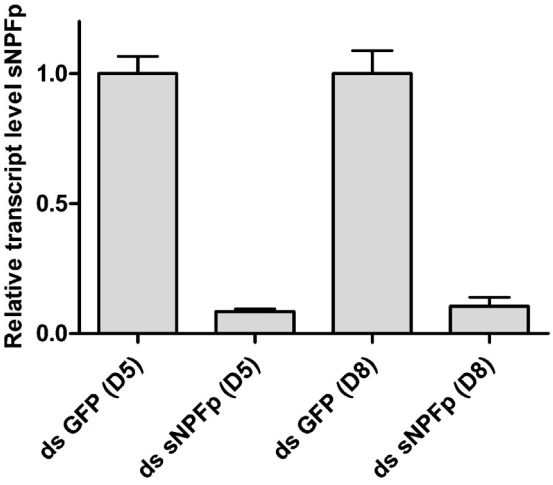

Fig. 4. Relative *Schgr*-sNPF precursor transcript levels in the brain upon *Schgr*-sNPFp knockdown. Locusts were injected with 200 ng dsRNA corresponding to either GFP or sNPFp. Injection of sNPFp dsRNA resulted in a 90% reduction in transcript levels five days after injection. Similar transcript levels were maintained until at least eight days after injection. Data represent mean values ± SEM ($n = 6$).

reduction in *Schgr*-sNPFp transcript levels was shown to persist until at least eight days after injection (Fig. 4). When the animals were subjected to a feeding assay five days after dsRNA injection, locusts in which the *Schgr*-sNPFp specific mRNA was silenced, showed a highly significant (approximately fourfold) increase in food-uptake compared to control animals (Fig. 5).

4. Discussion

The identified sequence displays all characteristics that are typical for a neuropeptide precursor [31]. All necessary elements for the formation of mature *Schgr*-sNPF peptides are therefore present (Fig. 1). Since arthropod genomes typically contain only a single sNPF-encoding gene, these results indicate that no other *Schgr*-sNPF isoforms are likely to be present, apart from the two peptides previously identified in mass spectrometric studies [8,22]. At present, it is not clear whether, in addition to sNPFs, other bioactive peptides might be encoded in this precursor. It is interesting, however, to notice some conserved amino acid sequence pattern (starting with SDP) in the adjacent sequences following the amidation signal of the sNPFs. Although the function of these SDP-motifs remains unclear, they can also be observed in the sNPF precursors of *Triboleum castaneum* [GenBank: DAA34847.1], *Aedes aegypti* [Genbank: ABE72968.1], *Anopheles gambiae* [UniProt: A0SIX6.2], and *D. melanogaster* [RefSeq: NP_724239.1].

Based on the structure of the *Schgr*-sNPFp sequence, an update of nomenclature seems appropriate. At the time of discovery of the *Schgr*-sNPF peptides, Clynen et al. were unable to ascertain whether the observed peptides were genuine sNPF isoforms or whether the shorter *Schgr*-sNPF$^{4\text{-}11}$ (SPSLRLRFa) was derived from the larger *Schgr*-sNPF (SNRSPRLRLRFa). These peptides were therefore named emphasizing their overlap in amino acid sequence: *Schgr*-sNPF and *Schgr*-sNPF$^{4\text{-}11}$ [8]. The identification of the *Schgr*-sNPF precursor, however, has shown that both *Schgr*-sNPF and *Schgr*-sNPF$^{4\text{-}11}$ are separately encoded in the precursor and that both peptides are genuine *Schgr*-sNPF isoforms. Therefore, we recommend adjusting the names of the peptides from *Schgr*-sNPF and *Schgr*-sNPF$^{4\text{-}11}$ to *Schgr*-sNPF-1 and -2 respectively, according to their order of appearance in the precursor sequence. This way, the structure of the peptide precursor is taken into account and nomenclature is adjusted to correspond to that used in other insect species, such as *D. melanogaster* (*Drome*-sNPF-1 to -4) and *A. mellifera* (*Apime*-sNPF-1 and -2) [12,31].

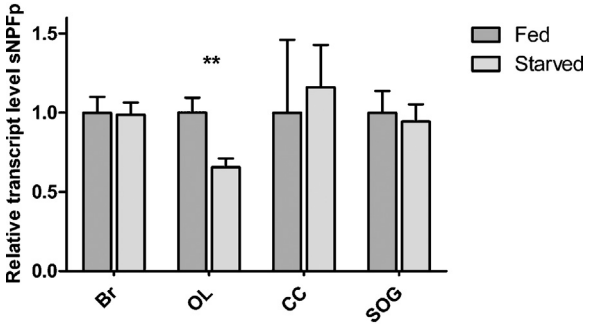

Fig. 3. Relative *Schgr*-sNPF precursor transcript levels upon starvation. Expression levels of the Schgr-sNPFp transcript in the optic lobes were significantly lower after five days of starvation. Br: brain; OL: optic lobes; CC: corpora cardiaca; and SOG: suboesophageal ganglion. Data represent mean values ± SEM ($n = 9$). Results were analyzed using Student's *t*-test. $^{**}p < 0.005$.

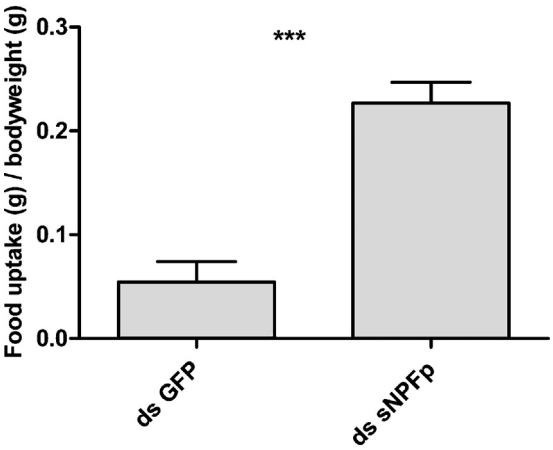

Fig. 5. RNAi mediated knockdown of the *Schgr*-sNPF precursor transcript and its effect on food uptake. Silencing of the *Schgr*-sNPFp mRNA yields a fourfold augmentation of food uptake in non-starved adult locusts. Data represent mean values ± SEM ($n = 15$). Results were analyzed using Student's *t*-test. $^{***}p < 0.0001$.

The main function of sNPF in arthropod physiology appears to be the regulation of feeding, a highly complex process regulated by numerous peptides, as discussed in recent studies [1,29,30,33] and reviewed by Spit et al. [26]. Although sNPF's effects on feeding are evident in the vast majority of studies, the direction of the effect appears to differ between insect species. In *D. melanogaster*, *Bombyx mori*, *A. mellifera*, *Periplaneta americana*, and *Leptinotarsa decemlineata*, sNPF signaling either stimulates feeding and food-search, or is correlated with a physiological state in which the animals more actively engage in food-search behavior [13,15,19,21]. In *Aedes aegypti* and *S. invicta*, however, sNPF does not seem to follow this positive correlation with feeding [4,6]. More conclusive evidence of sNPF's inhibitory role in feeding came when the nucleotide sequence encoding the sNPF receptor was identified in *S. gregaria* [3]. RNAi mediated knockdown of this receptor significantly stimulated food uptake, while injection of either of the two *Schgr*-sNPF isoforms resulted in a potent reduction of feeding [10]. In the present study, we elaborate on these results with the identification of the nucleotide sequence encoding the *Schgr*-sNPF precursor and with the finding that silencing this precursor mRNA strongly stimulates food uptake in this locust species. *Schgr*-sNPFp transcript levels in the brain significantly declined by injecting a fragment of double stranded RNA, corresponding to a 256 bp section of the *Schgr*-sNPFp cDNA. This procedure generated a potent transcript knockdown of over 90% (Fig. 4). When food uptake was analyzed in these animals, we observed an increase of the ingested amount of food of more than 300% (Fig. 5). These results provide new evidence for an inhibitory role of *Schgr*-sNPF in the regulation of feeding behavior in *S. gregaria*, although it remains mostly unclear how these effects are established exactly.

The distribution of the *Schgr*-sNPFp transcript appeared to be limited to tissues of neural origin, while its expression was very low or nearly undetectable in male and female gonads and flight muscles (Fig. 2). In this aspect, the *Schgr*-sNPFp transcript distribution resembles that of the sNPF peptides in *D. melanogaster* [15] and *Reticulitermes flavipes* [23], and of the *Schgr*-sNPF receptor [10]. In several insect species, such as *Bombyx mori* and *Aedes aegypti*, sNPF expression levels have been shown to decline in starved animals compared to non-starved controls [4,20]. Similar results have previously also been obtained for the sNPF receptors of *S. invicta* and *S. gregaria* [6,10]. While no effects of starvation were observed in the central mass of the locust brain, the suboesophegeal ganglion or the *corpora cardiaca*, starvation during a period of five days significantly reduced *Schgr*-sNPFp transcript levels in the optic lobes, the primary visual centers of the brain (Fig. 3). Interestingly, in *D. melanogaster*, starvation was shown to induce *Drome*-sNPF expression in sensory receptor neurons in the antennal lobes of the brain, which are responsible for the processing and propagation of olfactory stimuli. It was shown that sNPF signaling in these neurons plays a key role in starvation-dependent behavior and the authors argued that sNPF expression might modulate the organism's receptivity to external olfactory cues [25]. Analogous to these results, but bearing in mind that in *S. gregaria* sNPF has inhibitory rather than stimulatory effects on feeding, the observed starvation-dependent decrease of sNPFp transcript levels in the optic lobes may also be indicative for a transition to a physiological state more receptive to sensory (*i.c.* visual) stimuli, thereby facilitating food-search and feeding behaviors.

In conclusion, we have shown that both *Schgr*-sNPF and *Schgr*-sNPF$^{4-11}$ are genuine, precursor-encoded *Schgr*-sNPF isoforms and therefore suggest an update of their nomenclature to *Schgr*-sNPF-1 and -2 respectively. Furthermore, we have generated more evidence supporting an inhibitory role of sNPF in the control of feeding in *S. gregaria*. Although the exact mode of action remains largely unknown, the observation that sNPFp transcript levels in the optic lobes decrease upon starvation may suggest that *Schgr*-sNPF can modulate the animal's receptivity to visual cues, thereby affecting food searching behavior.

Acknowledgments

The authors would like to thank Roger Jonckers for taking care of the locust breeding facilities and Joost Van Duppen and Michiel Holtof for technical support. We also gratefully acknowledge the Interuniversity Attraction Poles programs (Belgian Science Policy Grant (P7/40)), the Research Foundation of Flanders (FWO-Flanders) and the K.U. Leuven Research Foundation (GOA/11/02) for financial support.

References

[1] Al-Anzi B, Armand E, Nagamei P, Olszewski M, Sapin V, Waters C, et al. The leucokinin pathway and its neurons regulate meal size in *Drosophila*. Curr Biol 2010;20:969–78.
[2] Ament SA, Velarde RA, Kolodkin MH, Moyse D, Robinson GE. Neuropeptide Y-like signalling and nutritionally mediated gene expression and behaviour in the honey bee. Insect Mol Biol 2011;20:335–45.
[3] Badisco L, Huybrechts J, Simonet G, Verlinden H, Marchal E, Huybrechts R, et al. Transcriptome analysis of the desert locust central nervous system: production and annotation of a *Schistocerca gregaria* EST database. PLoS ONE 2011;6:e17274, http://dx.doi.org/10.1371/journal.pone.0017274.
[4] Brown MR, Klowden MJ, Crim JW, Young L, Shrouder LA, Lea AO. Endogenous regulation of mosquito host-seeking behavior by a neuropeptide. J Insect Physiol 1994;40:399–406.
[5] Cerstiaens A, Benfekih L, Zouiten H, Verhaert P, De LA, Schoofs L. Led-NPF-1 stimulates ovarian development in locusts. Peptides 1999;20:39–44.
[6] Chen ME, Pietrantonio PV. The short neuropeptide F-like receptor from the red imported fire ant, *Solenopsis invicta* Buren (Hymenoptera: Formicidae). Arch Insect Biochem Physiol 2006;61:195–208.
[7] Chen W, Shi W, Li L, Zheng Z, Li T, Bai W, et al. Regulation of sleep by the short neuropeptide F (sNPF) in *Drosophila melanogaster*. Insect Biochem Mol Biol 2013;43:809–19.
[8] Clynen E, Husson SJ, Schoofs L. Identification of new members of the (short) neuropeptide F family in locusts and *Caenorhabditis elegans*. Trends Comp Endocrinol Neurobiol 2009;1163:60–74.
[9] Clynen E, Schoofs L. Peptidomic survey of the locust neuroendocrine system. Insect Biochem Mol Biol 2009;39:491–507.
[10] Dillen S, Zels S, Verlinden H, Spit J, Van Wielendaele P, Vanden Broeck J. Functional characterization of the short neuropeptide F receptor in the desert locust, *Schistocerca gregaria*. PLoS ONE 2013;8:e53604, http://dx.doi.org/10.1371/journal.pone.0053604.
[11] Gade G, Goldsworthy GJ. Insect peptide hormones: a selective review of their physiology and potential application for pest control. Pest Manag Sci 2003;59:1063–75.
[12] Hummon AB, Richmond TA, Verleyen P, Baggerman G, Huybrechts J, Ewing MA, et al. From the genome to the proteome: uncovering peptides in the *Apis* brain. Science 2006;314:647–9.
[13] Huybrechts J, De Loof A, Schoofs L. Diapausing Colorado potato beetles are devoid of short neuropeptide F I and II. Biochem Biophys Res Commun 2004;317:909–16.
[14] Lee KS, Kwon OY, Lee JH, Kwon K, Min KJ, Jung SA, et al. Drosophila short neuropeptide F signalling regulates growth by ERK-mediated insulin signalling. Nat Cell Biol 2008;10, 468-U230.
[15] Lee KS, You KH, Choo JK, Han YM, Yu K. Drosophila short neuropeptide F regulates food intake and body size. J Biol Chem 2004;279:50781–9.
[16] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. Methods 2001;25:402–8.
[17] Marchal E, Verlinden H, Badisco L, Van Wielendaele P, Vanden Broeck J. RNAi-mediated knockdown of Shade negatively affects ecdysone-20-hydroxylation in the desert locust, *Schistocerca gregaria*. J Insect Physiol 2012;58:890–6.
[18] Marciniak P, Szymczak M, Rogalska L, Rosinski G. Developmental and myotropic effects of the Led-NPF-I peptide in tenebrionid beetles. Invertebr Reprod Dev 2013:1–7.
[19] Mikani A, Wang QS, Takeda M. Brain-midgut short neuropeptide F mechanism that inhibits digestive activity of the American cockroach, *Periplaneta americana* upon starvation. Peptides 2012;34:135–44.
[20] Nagata S, Matsumoto S, Nakane T, Ohara A, Morooka N, Konuma T, et al. Effects of starvation on brain short neuropeptide F-1, -2, and -3 levels and short neuropeptide F receptor expression levels of the silkworm, *Bombyx mori*. Front Endocrinol (Lausanne) 2012;3:3.
[21] Nagata S, Morooka N, Matsumoto S, Kawai T, Nagasawa H. Effects of neuropeptides on feeding initiation in larvae of the silkworm, *Bombyx mori*. Gen Comp Endocrinol 2011;172:90–5.
[22] Nässel DR, Wegener C. A comparative review of short and long neuropeptide F signaling in invertebrates: any similarities to vertebrate neuropeptide Y signaling. Peptides 2011;32:1335–55.

[23] Nuss AB, Forschler BT, Crim JW, Brown MR. Distribution of neuropeptide F-like immunoreactivity in the eastern subterranean termite, *Reticulitermes flavipes*. J Insect Sci 2008;8:1–18.
[24] Ons S, Sterkel M, Diambra L, Urlaub H, Rivera-Pomar R. Neuropeptide precursor gene discovery in the Chagas disease vector *Rhodnius prolixus*. Insect Mol Biol 2011;20:29–44.
[25] Root CM, Ko KI, Jafari A, Wang JW. Presynaptic facilitation by neuropeptide signaling mediates odor-driven food search. Cell 2011;145:133–44.
[26] Spit J, Badisco L, Verlinden H, Van Wielendaele P, Zels S, Dillen S, et al. Peptidergic control of food intake and digestion in insects. Can J Zool 2012;90:489–506.
[27] Tobback J, Boerjan B, Vandersmissen HP, Huybrechts R. Male reproduction is affected by RNA interference of period and timeless in the desert locust *Schistocerca gregaria*. Insect Biochem Mol Biol 2012;42:109–15.
[28] Tobback J, Vuerinckx K, Boerjan B, Huybrechts R. RNA interference mortality points to noncircadian functions for the clock gene in the desert locust *Schistocerca gregaria*. Insect Mol Biol 2012;21:369–81.
[29] Van Wielendaele P, Dillen S, Zels S, Badisco L, Vanden Broeck J. Regulation of feeding by neuropeptide F in the desert locust, *Schistocerca gregaria*. Insect Biochem Mol Biol 2013;43:102–14.
[30] Van Wielendaele P, Dillen S, Marchal E, Badisco L, Vanden Broeck J. CRF-like diuretic hormone negatively affects both feeding and reproduction in the desert locust, *Schistocerca gregaria*. PLoS ONE 2012;7:e31425, http://dx.doi.org/10.1371/journal.pone.0031425.
[31] Vanden Broeck J. Neuropeptides and their precursors in the fruitfly, *Drosophila melanogaster*. Peptides 2001;22:241–54.
[32] Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 2002;3, http://dx.doi.org/10.1186/gb-2002-3-7-research0034, research0034–research0034.11.
[33] Wei Z, Baggerman G, Nachman RJ, Goldsworthy G, Verhaert P, De Loof A, et al. Sulfakinins reduce food intake in the desert locust, *Schistocerca gregaria*. J Insect Physiol 2000;46:1259–65.
[34] Wynant N, Verlinden H, Breugelmans B, Simonet G, Vanden Broeck J. Tissue-dependence and sensitivity of the systemic RNA interference response in the desert locust, *Schistocerca gregaria*. Insect Biochem Mol Biol 2012;42:911–7.